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(54) **SODIUM/PROTON COUNTERTRANSPORTER GENE**

(57) The present inventors successfully cloned the  
rice Na<sup>+</sup>/H<sup>+</sup> antiporter gene. It is possible to produce salt

tolerant plants by using the isolated gene, or genes with  
equivalent functions.

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## Description

## Technical Field

- 5 [0001] The present invention relates to a novel Na<sup>+</sup>/H<sup>+</sup> antiporter derived from plants and the DNA encoding the antiporter, as well as methods for producing and using the same.

## Background Art

- 10 [0002] Salt tolerance of plants is important to both agriculture and environmental protection. Today, one third of the land on earth is said to be dry land. Further, it is anticipated that the proportion of dry land will increase in the future, due to the progressive desertification of both cultivated land and green land. Considering the prediction that the world population in the year 2050 will be 1.5 times that of today and the serious problems of provisions arising as a result, development of cultivars that grow on land ill-fitted for cultivation, especially on dry land, as well as cultivation techniques  
 15 for the same is a matter of great urgency. The problem with agriculture on dry land is salt accumulation. In a dry climate, evapotranspiration outstrips precipitation and continued irrigation on land where much is desired for drainage leads to plenty of salt accumulation, due to the deposition of salt on the surface by acceleration of rise in subterranean water level that bear salinity. Examples where cultivation becomes impossible as a result are known from the ancient past, represented by the end of Tigris-Euphrates civilization. The problem still arises today. Thus, innovation of agriculture, on dry land and on land where salt is accumulated, to enhance the salt tolerance of plants is of great importance  
 20 (Toshiaki Tanno (1983) Kagaku to Seibutsu 21:439-445 "Salt tolerance of crops and mechanism of the same"; Yasutaka Uchiyama (1988) Kagaku to Seibutsu 26:650-659 "Agricultural use of salinenvironment").

- [0003] There are two kinds of stress related to salt stress against plants, namely stress by osmotic pressure and stress by ionicity. An osmotic pressure stress is a stress whose action is the same as the stress by dehydration. It results from high osmotic pressure, due to high salinity environment around the plant, which leads to a setback of water  
 25 absorption of the plant and at the same time deprivation of water from the plant body. It is known that a mechanism exists in the plant to avoid the osmotic pressure stress. The core substances associated with this function are ions (such as K<sup>+</sup>, Na<sup>+</sup>, Cl<sup>-</sup>, organic acid, etc.) as well as substances called compatible solutes. The term "compatible solute" refers to substances such as sugar, proline (a kind of amino acid), and glycine betaine (a quaternary ammonium compound), and so on, which do not disturb the metabolic pathway or inhibit enzymatic action, even when accumulated at a high concentration in the cell. Plant cells accumulate these substances which, in turn, preserve the osmotic pressure balance to the external world (Manabu Ishitani, Keita Arakawa, and Tetsuko Takabe (1990) Chemical Regulation of Plants 25:149-162, "Molecular mechanism of salt tolerance in plants").

- [0004] Almost no development has been made regarding the mechanism of plants to avoid ionic stress. Absorption of excess Na<sup>+</sup> by the plant cell leads to inhibition of intracellular enzyme reaction and finally to metabolic trouble (Toru Matoh (1997) Chemical Regulation of Plants 32:198-206, "Salt tolerance mechanism of the plant"). Therefore, it is necessary to eliminate the intracellular accumulated Na<sup>+</sup> from the cell or isolate it into intracellular organs, such as vacuoles. The Na<sup>+</sup>/H<sup>+</sup> antiporter (sodium/proton antiporter) is assumed to play the central role in this process. The Na<sup>+</sup>/H<sup>+</sup> antiporters of plant cells are thought to exist on both the cell membrane and the vacuolar membrane. They  
 40 utilize the pH gradient formed between the biomembranes by the H<sup>+</sup> pump (H<sup>+</sup>-ATPase and H<sup>+</sup>-PPase), an element that transports H<sup>+</sup> as the energy to transport Na<sup>+</sup> existing in the cytoplasm out of the cell or into the vacuole. Moreover, it is presumed that plants contacted with salt of high density, have to retain intercellular K<sup>+</sup>/Na<sup>+</sup> ratio high enough, maintaining the osmotic pressure balance between the cell exterior and interior by accumulating Na<sup>+</sup> in the vacuole through the Na<sup>+</sup>/H<sup>+</sup> antiporter.

- 45 [0005] The Na<sup>+</sup>/H<sup>+</sup> antiporters found existing on plasma membrane are well examined in animals, yeasts, bacteria and so on. On the plasma membrane of an animal cell, H<sup>+</sup> is carried by the Na<sup>+</sup>/H<sup>+</sup> antiporter, to maintain the balance of H<sup>+</sup> in the cell, utilizing the Na<sup>+</sup> concentration gradient between the membranes formed by Na<sup>+</sup>/K<sup>+</sup>-ATPase. Therefore, the antiporter is presumed to be deeply related with intracellular pH modulation, control of the cell volume, as well as Na<sup>+</sup> transport through the plasma membrane (Orlowski, J. and Grinstein, S. (1997) J.Biol.Chem. 272:22373-22376; Aronson, P.S. (1985) Ann.Rev.Physiol. 47:545-560). Na<sup>+</sup>/H<sup>+</sup> antiporters exist in various cells of animals and six isoforms (NHE 1 to 6) have been reported (Orlowski, J. and Grinstein, S. (1997) J.Biol.Chem. 272:22373-22376).  
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- [0006] The first gene cloned for yeast was the gene (sod2) from fission yeast (*Schizosaccharomyces pombe*), which was cloned as a gene related to Na<sup>+</sup> transport and salt tolerance (Jia, Z.P., McCullough, N., Martel, R., Hemmingsen, S. and Young, P.G. (1992) EMBO J. 11:1631-1640). Also, a gene with high identity to this gene has been found from a budding yeast (*Saccharomyces cerevisiae*), as well as *Zygosaccharomyces rouxii* (named NHA1 and ZSOD2, respectively) (Prior, C. et al. (1996) FEBS Letter 387:89-93; Watanabe, Y. et al. (1995) Yeast 11:829-838). Two different Na<sup>+</sup>/H<sup>+</sup> antiporter genes (nhaA, nhaB) have been isolated from *E.coli* (Karpel, R. et al. (1988) J.Biol.Chem. 263:10408-10410; Pinner, E. et al. (1994) J.Biol.Chem. 269:26274-26279), each closely related to Na<sup>+</sup> transport and salt  
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tolerance. With respect to plants, activities in algae and such have been examined (Katz, A. *et al.* (1989) *Biochim. Biophys. Acta* 983:9-14).

[0007] On the other hand, there are only reports on activity in plants for antiporters restricted on vacuolar membranes. To date,  $\text{Na}^+/\text{H}^+$  antiporters on the vacuoles have been investigated in connection with salt tolerance in halophytes growing in an environment with high salinity (Matoh, T. *et al.* (1989) *Plant Physiol.* 89:180-183; Hassidim, M. *et al.* (1990) *Plant Physiol.* 94:1795-1801; Barkla, B.J. *et al.* (1995) *Plant Physiol.* 109:549-556), as well as in glycophytes with high salt tolerance, like barley and sugar beet (Hassidim, M. *et al.* (1990) 94:1795-1801; Blumwald, E. *et al.* (1987) *Plant Physiol.* 85:30-33; Garbarino, J. and DuPont, F.M. (1988) *Plant Physiol.* 86:231-236; Garbarino, J. and DuPont, F.M. (1989) *Plant Physiol.* 89:1-4; Staal, M. *et al.* (1991) *Physiol. Plant.* 82:179-184). The above findings indicate that  $\text{Na}^+/\text{H}^+$  antiporters are closely related to salt tolerance of plants. There are several reports on characteristics of  $\text{Na}^+/\text{H}^+$  antiporters on the vacuolar membrane. The  $K_m$  of the antiporter activity for  $\text{Na}^+$  is about 10mM similar to that on cytomembrane of mammals (Blumwald, E. *et al.* (1987) *Plant Physiol.* 85:30-33; Garbarino, J. and DuPont, F.M. (1988) *Plant Physiol.* 86:231-236; Orlowski, J. (1993) *J. Biol. Chem.* 268:16369-16377). Moreover, it is known that amiloride and amiloride derivatives, which are specific inhibitors of  $\text{Na}^+$  transporters, inhibit the  $\text{Na}^+/\text{H}^+$  antiporters on the plant vacuolar membrane and that on the mammalian plasma membrane in a competitive manner (Blumwald, E. *et al.* (1987) *Plant Physiol.* 85:30-33; Orlowski, J. (1993) *J. Biol. Chem.* 268:16369-16377; Tse, C.M. *et al.* (1993) *J. Biol. Chem.* 268:11917-11924; Fukuda, A. *et al.* (1998) *Plant Cell Physiol.* 39:196-201). These findings suggest the characteristic similarities between  $\text{Na}^+/\text{H}^+$  antiporter on the vacuolar membrane of plants and that on mammalian plasma membrane. There are various reports on  $\text{Na}^+/\text{H}^+$  antiporter activity of plants as mentioned above, however, in spite of the various trials done, analysis of the substantial part, namely genes as well as proteins thereof, were still left behind (Katz, A. *et al.* (1989) *Biochim. Biophys. Acta* 983:9-14; Barkla, B. and Blumwald, E. (1991) *Proc. Natl. Acad. Sci. USA* 88:11177-11181; Katz, A., Kleiman, T.R., and Pick, U. (1994) *Biochemistry* 33:2389-2393).

[0008] Recently, a gene expected to encode a protein that shares amino acid sequence homology with known  $\text{Na}^+/\text{H}^+$  antiporter has been cloned from *Arabidopsis*; however, the function of this gene remains to be resolved (M.P. Apse *et al.* (1998) Final Programme and Book of Abstracts "11th International Workshop on Plant Membrane Biology", Springer; C.P. Darley *et al.* (1998) Final Programme and Book of Abstracts "11th International Workshop on Plant Membrane Biology", Springer).

[0009] Examples of  $\text{Na}^+/\text{H}^+$  antiporter genes isolated from plants are only those isolated from *Arabidopsis*, a dicotyledon, described above. No isolation of genes from monocotyledoneae, including species such as rice and maize, which are industrially useful crops, have been reported until now.

#### Disclosure of the Invention

[0010] Of all the important crops, rice is a crop with low salt tolerance. Its growth is inhibited to the halves with 150mM NaCl as compared to barley, which is a highly salt tolerant crop, and shows inhibition of the same level with 250 mM NaCl. Garbarino *et al.* reported the suppression of  $\text{Na}^+$  flow to the shoot by accumulating  $\text{Na}^+$  in the vacuole of the root might increase salt tolerance of barleys (Garbarino, J. and DuPont, F.M. (1988) *Plant Physiol.* 86:231-236). From verifying this fact, it has been known that the  $\text{Na}^+/\text{H}^+$  antiporter activity of the barley root vacuolar membrane increases through treatment with salt. It has also been known that barley has far and away a higher activity than rice (Garbarino, J. and DuPont, F.M. (1988) *Plant Physiol.* 86:231-236; Fukuda, A. Yazaki, Y., Ishikawa, T., Koike, S., and Tanaka, Y. (1998) *Plant Cell Physiol.* 39:196-201).

[0011] On the contrary, the activity does not rise in rice even if it is treated with salt (Fukuda, A. Yazaki, Y., Ishikawa, T., Koike, S., and Tanaka, Y. (1998) *Plant Cell Physiol.* 39:196-201). Further,  $\text{Na}^+$  transport from root to the shoot of rice is known to be higher than that of the phragmites, which belong to the *Gramineae* family, like rice, and shows higher salt tolerance (Matsushita, N. and Matoh, T. (1991) *Physiol. Plant.* 83:170-176). Therefore, it is possible that the strength of  $\text{Na}^+/\text{H}^+$  antiporter activity of the root vacuolar membrane is deeply associated with rice salt tolerance. These reports indicate that it might be possible to increase salt tolerance of rice by rising  $\text{Na}^+/\text{H}^+$  antiporter activity in the rice root. On this account, there was a desire to isolate genes that might increase  $\text{Na}^+/\text{H}^+$  antiporter activity of rice.

[0012] This situation led to the present invention, an object of which is to provide an  $\text{Na}^+/\text{H}^+$  antiporter derived from monocotyledoneae, preferably rice, and gene(s) encoding the same, as well as a method for producing and using the same. The present invention provides use of the gene for production of salt tolerant plants as a favorable use of the present DNA.

[0013] The present inventors identified a cDNA clone from rice anthotaxy that shares homology with the  $\text{Na}^+/\text{H}^+$  antiporter (*NHX1*) gene from the budding yeast by analyzing a base sequence from the GeneBank higher plants database. Using this sequence as a probe, the present inventors succeeded in newly cloning the full-length gene designated "*OsNHX1*", which is expected to encode the  $\text{Na}^+/\text{H}^+$  antiporter of rice.

[0014] The isolated *OsNHX1* cDNA is approximately 2.3kb and is presumed to encode a protein of 535 amino acids (Figure 1). From an amino acid hydrophobicity analysis, the protein was detected to have 12 transmembrane regions

(Figure 2).

**[0015]** The amino acid sequence predicted from *OsNHX1* was detected to have significant identity with the amino acid sequence of NHX1 and mammalian Na<sup>+</sup>/H<sup>+</sup> antiporter (NHE) (Table 1). Specifically, high identity was seen in the transmembrane region supposed to be involved in ion transport (Figure 3).

**[0016]** These three proteins (NHX1 from budding yeast, NHE6 from mammals, and *OsNHX1*) turned out to form a cluster, according to the dendrogram formed for various Na<sup>+</sup>/H<sup>+</sup> antiporters reported to date (Figure 4). The *OsNHX1* protein of the present invention is expected to be expressed in intracellular organs, such as vacuoles, and play an important role in the Na<sup>+</sup> transport of the vacuolar membrane, due to the report that NHX1 protein is expressed in the late endosome (Nass, R. and Rao, R. (1998) *J.Biol.Chem.* 273:21054-21060) and the indication that NHE6 protein is also expressed in the cell (Numata, M., Petrecca, K., Lake, N. and Orlowski J. (1998) *J.Biol.Chem.* 273:6951-6959).

**[0017]** Further, the present inventors succeeded in obtaining transgenic plants by transferring the isolated *OsNHX1* gene into the rice callus and redifferentiating them utilizing Agrobacterium method.

**[0018]** The present invention relates to a novel Na<sup>+</sup>/H<sup>+</sup> antiporter derived from monocotyledoneae and the DNA coding said antiporter, as well as methods for production and use, especially for the production of salt tolerant plants using same. More specifically, the present invention provides the following:

(1) a DNA selected from the group consisting of:

- (a) a DNA encoding the protein consisting of the amino acid sequence described in SEQ ID NO: 2, and
- (b) a DNA comprising the coding region of the base sequence described in SEQ ID NO: 1;

(2) a DNA encoding the Na<sup>+</sup>/H<sup>+</sup> antiporter derived from monocotyledoneae selected from the group consisting of:

- (a) a DNA encoding the protein consisting of the amino acid sequence described in SEQ ID NO:2, wherein one or more amino acids are substituted, deleted, inserted and/or added, and
- (b) a DNA hybridizing under a stringent conditions to the DNA consisting of the base sequence described in SEQ ID NO:1;

(3) the DNA of (2), wherein the monocotyledoneae is a plant belonging to the Gramineae family;

(4) a vector comprising the DNA of (1) or (2);

(5) a transformant cell having the DNA of (1) or (2), or the vector of (4);

(6) the transformant cell of (5), wherein the cell is a plant cell;

(7) a protein encoded by the DNA of (1) or (2);

(8) a method for production of the protein of (7), which comprises the steps of:

cultivating the transformant cell of (5), and

recovering the expressed protein from said cell or the supernatant of the culture thereof;

(9) a transformant plant comprising the transformant cell of (6);

(10) the transformant plant of (9), wherein the plant is a monocotyledon;

(11) the transformant plant of (10), wherein the plant is a plant belonging to the *Gramineae* family;

(12) the transformant plant of (11), wherein the plant is rice;

(13) a transformant plant that is the offspring or clone of the transformant plant of any of (9) to (12);

(14) a material for the breeding of the transformant plant of any of (9) to (13);

(15) an antibody that binds to the protein of (7);

(16) a nucleic acid molecule that hybridizes with the DNA described in SEQ ID NO: 1, and which has a chain length of at least 15 nucleotides.

**[0019]** The present invention provides a novel Na<sup>+</sup>/H<sup>+</sup> antiporter derived from monocotyledoneae, as well as a DNA encoding the same. The base sequence of the cDNA encoding the Na<sup>+</sup>/H<sup>+</sup> antiporter "*OsNHX1*", derived from rice and isolated by the present inventors, is indicated in SEQ ID NO: 1. The amino acid sequence of the protein encoded by the cDNA is described in SEQ ID NO: 2.

**[0020]** The "*OsNHX1*" gene showed significant identity with many known amino acid sequences of the Na<sup>+</sup>/H<sup>+</sup> antiporters, and especially high identity was observed at sites related to ion transport. This finding suggests that "*OsNHX1*" protein plays an important role in Na<sup>+</sup> transport in rice. It is supposed that Na<sup>+</sup>/H<sup>+</sup> antiporters of plants are involved in the securement of osmotic pressure balance in the plant body under a high salinity stress. Thus, it is anticipated that the "*OsNHX*" gene especially can be applied to production of salt tolerant cultivars.

**[0021]** Not only "*OsNHX1*" protein, but also proteins with equivalent functions, are included in this invention. The

term "proteins with equivalent functions to 'OsNHX1' protein" herein means that the object protein functions as an Na<sup>+</sup>/H<sup>+</sup> antiporter. The activity of an Na<sup>+</sup>/H<sup>+</sup> antiporter can be detected, for example, by detecting the H<sup>+</sup> ejection from the biomembrane vesicle due to addition of Na<sup>+</sup> as the recovery of fluorescence, by monitoring H<sup>+</sup> concentration gradient between isolated biomembrane vesicle formed by H<sup>+</sup>-ATPase as the fluorescence extinction of acridine orange (Fukuda, A., Yazaki, Y., Ishikawa, T. Koike, S., and Tanaka, Y. (1998) *Plant Cell Physiol.* 39:196-201).

**[0022]** In one embodiment, the protein with equivalent function to "OsNHX1" is a mutant protein having amino acid sequence with one or more amino acid substitution, deletion, insertion and/or addition to the amino acid sequence of "OsNHX1" protein (SEQ ID NO: 2), and which retains equivalent functions with "OsNHX1" protein. Such proteins can be prepared, for example, according to the following method. A method inducing mutations in the amino acid of "OsNHX1" can be mentioned as one method well known to ordinary skilled in the art. That is, one ordinary skilled in the art can prepare a modified protein with equivalent functions to "OsNHX1" by modifying the amino acid sequence of "OsNHX1" protein (SEQ ID NO: 2). For example, by utilizing a site-directed mutagenesis method (Kramer, W. & Fritz, H. -J. "Oligonucleotide-directed construction of mutagenesis via gapped duplex DNA" *Methods in Enzymology* 154:350-367, 1987) and such with the purpose to increase protein activity or the like. Mutations of amino acids also happen to occur in nature. The protein of this invention include proteins having amino acid sequence with 1 or more amino acids substitution, deletion, insertion or addition to the natural amino acid sequence of "OsNHX1" protein (SEQ ID NO: 2), and that retain equivalent functions to natural proteins, regardless whether they are artificial or derived from nature. There is no limitation on the part or the number of amino acid in the protein to be modified, so long as the modified protein retains equivalent functions with the natural "OsNHX1" protein. Generally, amino acid modifications are done to amino acids within 100 amino acids, preferably within 50 amino acids, much more preferably within 20 amino acids, and most preferably within 5 amino acids.

**[0023]** In another embodiment, the protein having equivalent functions with "OsNHX1" protein is a protein encoded by a DNA derived from monocotyledoneae that hybridizes to the DNA encoding "OsNHX1" protein (SEQ ID NO: 1), having an equivalent function with "OsNHX1" protein. Techniques such as hybridization techniques (Southern, E.M. : *Journal of Molecular Biology*, Vol.98, 503, 1975) and polymerase chain reaction (PCR) techniques (Saiki, R.K. *et al. Science*, Vol.230, 1350-1354, 1985; Saiki, R.K. *et al. Science*, Vol.239, 487-491, 1988) can be mentioned as techniques known to those skilled in the art for preparing proteins. That is, it is routine for a person skilled in the art to isolate a DNA with high identity to the "OsNHX1" gene from rice or other monocotyledon and obtain proteins with an equivalent function to "OsNHX1" protein from that DNA, using the base sequence of "OsNHX1" gene (SEQ ID NO: 1) or parts thereof as a probe, and oligonucleotides hybridizing specifically to the base sequence of "OsNHX1" gene (SEQ ID NO: 1) as a primer. Such proteins, derived from monocotyledoneae with an equivalent function to the "OsNHX1" protein, obtainable by hybridization technique or PCR technique, are included in the proteins of this invention.

**[0024]** Monocotyledoneae, preferably plants belonging to the Gramineae family can be mentioned as plants used as the source of genes for isolation by hybridization techniques and PCR techniques. For example, besides rice, barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), maize (*Zea mays*) and so on can be mentioned, as plants belonging to the Gramineae family. However, it is not limited to them.

**[0025]** Methods for isolating DNA encoding proteins with an equivalent function to the "OsNHX1" protein using the above-described techniques include, for example, but are not limited to, the following. For example, hybridization of cDNA or genomic libraries, prepared from monocotyledoneae with probes (for example, DNA consisting of the base sequence described in SEQ ID NO: 1 or parts thereof) labeled with <sup>32</sup>P and such, is carried out. Conditions for hybridization using <sup>32</sup>P labeled probes are 25°C (without formamide) as a mild condition and usually 42°C, employing hybridization solutions (50% formamide, 5X SSPE, 2X Denhard's solution, 0.1% (w/v) SDS, and 100 µg/ml of herring sperm DNA (Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), 2<sup>nd</sup> Ed.)). Prehybridization is carried out at a minimum for more than an hour, and hybridization is performed for 24 hours. Washing of the hybridized filter is carried out at 25°C (wash solution: 2X SSC, 0.1%SDS) for a mild condition (a condition with low stringency), at 42°C (wash solution: 2X SSC, 0.1%SDS) for an ordinary condition, and at 56°C (wash solution: 0.1X SSC, 0.1%SDS) for a stringent condition (a condition with high stringency).

**[0026]** If the protein encoded by the DNA isolated as above has an equivalent function as "OsNHX1" protein, it generally shows a high amino acid sequence identity with "OsNHX1" protein. The term "high identity" as used herein refers to an identity higher than at least 60%, preferably higher than 80%, more preferably higher than 85%, and much more preferably higher than 90%. The amino acid sequence identity is calculated, for example, by a homology analysis program (Lipman, D.J. and Pearson, W.R. (1985) *Science* 227, 1435-1441) supplied by GENETYX software (Software development corporation).

**[0027]** The protein of the present invention can be prepared by methods known to those skilled in the art as recombinant proteins or as natural proteins. Recombinant proteins can be prepared, for example, by inserting a DNA encoding the protein of the present invention into an adequate expression vector, transfecting an appropriate cell with the vector and purifying the protein from the transformant cell, as described later on. Alternatively, natural proteins can be pre-

pared, for example, by exposing cell extracts, prepared from cells that express the protein of the present invention (for example, rice cells), to affinity columns to which antibodies, prepared by immunizing appropriate immune animals with prepared recombinant proteins or partial peptides thereof, are attached, and purifying the proteins bound to the column.

**[0028]** Additionally, the present invention provides DNAs encoding the proteins of the present invention described above. The DNAs of the present invention includes genomic DNAs, cDNAs, and chemosynthetic DNAs and so on, and can be any DNA without limitation, so long as it encodes a protein of the present invention. The base sequence of the "OsNHX1" cDNA, included in the present invention, is shown in SEQ ID NO: 1.

**[0029]** The genomic DNA, as well as the cDNA can be prepared according to conventional methods, known to those skilled in the art. Genomic DNA, for example, can be isolated using PCR, by designing appropriate primers from the base sequence information of the gene of the present invention, and then screening a genomic library using the obtained amplified DNA fragment as a probe. Alternatively, for example, it is possible to isolate the cDNA from a cDNA library according to the same manner.

**[0030]** The DNA of the present invention can be, for example, utilized in preparation of recombinant proteins, as well as in production of transformant plants with salt tolerance. In preparing recombinant proteins, generally, a DNA encoding the protein of the present invention is inserted into an appropriate expression vector, the expression vector is transferred into an appropriate cell, the transformed cell is cultivated and the expressed protein is purified.

**[0031]** Recombinant proteins can be prepared, for example, by transferring vectors, having DNAs encoding the protein of the present invention inserted therein, into cells, such as bacterial cells, like *E.coli*, yeast cells, insect cells, mammalian cells, and so on, by known gene transfer methods, like the electroporation method, the calcium phosphate transfection method and such, then expressing the recombinant proteins in the cell. Recombinant proteins expressed in the host cell can be purified according to methods known to those skilled in the art. For example, it is possible to express the protein as a fusion protein, with glutathione S-transferase (GST), using vectors such as pGEX (Pharmacia) in *E.coli*, and purify it using a glutathione column (Shigeo Ohno and Yoshifumi Nishimura (1997) "Cell Engineering supplement: Protocol of protein experiments" Shujun-sha).

**[0032]** Moreover, to prepare transformant plants using the DNA of the present invention, a DNA encoding the protein of the present invention is inserted into an appropriate vector, the vector is transferred into a plant cell, and the obtained transformed plant cell is regenerated. The transfer of the plant expression vector into the plant cell can be done for example, according to the species, through methods utilizing *Agrobacterium*s or methods involving the direct transfer into the cell. Methods that utilize *Agrobacterium*s, for example, are methods of Nagel *et al.* (*Microbiol.Lett.* 67:325 (1990)) and methods of Raineri *et al.* for rice (*Bio/Technology* 8:33-38(1990)). These are methods in which *Agrobacterium*s are transformed with plant expression vectors (pUC system and so on. For example, pCambia vector (Medical Research Council), etc.), and the transformed *Agrobacterium*s are transferred to plant cells using standard methods, like the leaf disk method, the callus method and so on. Methods for the directly transferring a plant expression vector into a cell include the electroporation method, the particle gun method, the calcium phosphate method, the polyethylene glycol method and so.

**[0033]** There is no limitation on the plant cells to which vectors of the invention may be transferred, but monocotyledonous, preferably plants belonging to the *Gramineae* family are mentioned. Plants, like maize except rice, can be mentioned as plants belonging to the *Gramineae* family. Incidentally, the "plant cell" of the present invention includes various forms of plant cells, such as suspension culture cells, protoplasts, a section from the leaf, callus, and so on.

**[0034]** For example, methods, like the callus differentiation method (Kyoizuka, J. and Shimamoto, K. (1991) *Plant Tissue Culture Manual*. Kluwer Academic Publishers, pp B1:1-16; Toki, S. (1997) *Plant Molecular Biology* 15:16-21), the differentiation method utilizing protoplasts (Shimamoto, K. *et al.* (1989) *Nature* 338:274-276; Kyoizuka, J. *et al.* (1987) *Mol.Gen.Genet.* 206:408-413), and such in response to the kind of plant used, can be utilized to regenerate transgenic plants from transgenic plant cells to which vectors are introduced.

**[0035]** Transgenic plants produced in this way show high Na<sup>+</sup>/H<sup>+</sup> antiporter activity as compared to wild-type plants, and are supposed to acquire salt tolerance thereby. Moreover, once a transformed plant transfected with the DNA of the present invention is obtained, it is possible to gain descendants from that plant body by syngensis or agamogenesis. Alternatively, plants can be mass-produced from breeding materials (for example, seeds, fruits, ears, tubers, tubercles, stubs, callus, protoplast, etc.) obtained from the plant, as well as descendants or clones thereof. Plant cells transferred with the DNA of the present invention, plant bodies including these cells, descendants and clones of the plant, as well as breeding materials obtained from the plant, its descendant and clones, are included in the present invention.

**[0036]** Such high Na<sup>+</sup>/H<sup>+</sup> antiporter activity as compared to wild-type plants can be achieved either by high expression of Na<sup>+</sup>/H<sup>+</sup> antiporter (change in quantity) or by expression of Na<sup>+</sup>/H<sup>+</sup> antiporter with higher activity (change in quality), or may be a result of both.

**[0037]** Further, the present invention provides antibodies binding to the proteins of the present invention described above. Both polyclonal antibodies and monoclonal antibodies are included in the present invention. Preparation of the antibody can be conducted according to methods known to those skilled in the art, for example, using methods of

Harlow *et al.* (Harlow, E. and Lane, D. (1988) Antibodies: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor; New York). Polyclonal antibodies can be obtained by injecting fusion proteins, synthesized in *E. coli* or synthesized peptides, into a rabbit as antigens, obtaining rabbit antiserum, and purifying antibodies therefrom by affinity chromatography. Monoclonal antibodies can be obtained by injecting antigens to mouse or rats, cloning and preparing hybridomas, and subjecting thus obtained antibody to affinity chromatography.

**[0038]** Furthermore, the present invention provides nucleic acid molecules that hybridize with the DNA encoding the protein of the present invention, and which have a chain length of at least 15 nucleotides. Such nucleic acid molecules can be used, for example, as probes to detect or isolate the DNA encoding the protein of the present invention, as well as primers to enhance such DNA. Such nucleic acid molecules preferably hybridize specifically to the DNA encoding the protein of the present invention. The term "hybridizes specifically" as used herein means that it hybridizes to the DNA encoding the protein of the present invention but it does not hybridize to DNAs encoding other proteins under a normal hybridization condition, preferably under a stringent condition for hybridization.

**[0039]** In addition, such nucleic acid molecules can be used as antisense oligonucleotides, ribozymes, and so on, that suppress expression of the protein of the present invention. Derivatives and modified forms of the antisense oligonucleotides can be used in the same manner as the antisense oligonucleotide itself. The antisense oligonucleotide does not have to be completely complementary to the nucleotides constituting the given region of the DNA or mRNA, and may include 1 or more nucleotide mismatches, provided it can suppress expression of the protein. An antisense oligonucleotide and a ribozyme that suppresses expression of a protein of the present invention can be a very useful tool for the function analysis of the protein of the present invention.

#### Brief Description of the Drawings

##### **[0040]**

Figure 1 shows the base sequence and the predicted amino acid sequence of the rice Na<sup>+</sup>/H<sup>+</sup> antiporter (*OsNHX1*) cDNA. The amino acid sequence is expressed in one letter notation.

Figure 2 shows the hydrophobicity plot of the amino acids of the *OsNHX1* protein. The abscissa indicates the amino acid residue, and the ordinate indicates the degree of hydrophobicity. Predicted transmembrane regions are shown as boxed numbers.

Figure 3 shows the amino acid sequence comparison between *OsNHX1* and other Na<sup>+</sup>/H<sup>+</sup> antiporters. Transmembrane regions (M3 to M6) are indicated above the sequence. Regarding the symbols under amino acids, "\*" represents that all amino acids are conserved; "." and "." represent that amino acid are similar. ":" indicates much more similarity than those indicated by ".". The box with A represents the binding site of the specific inhibitor, amiloride, and the boxes with B represent sites with high identities to the mammalian Na<sup>+</sup>/H<sup>+</sup> antiporter.

Figure 4 shows the result of phylogeny analysis of Na<sup>+</sup>/H<sup>+</sup> antiporter using ClustalX (Thompson, J.D. *et al.* (1994) *Nucleic Acids Research*, 22:4673-4680)(Neighbor Joining (NJ) method).

#### Best Mode for Carrying out the Invention

**[0041]** The present invention will be specifically explained with reference to the following examples. However, it should be noted that the present invention is not limited by these examples.

##### [Example 1] Cloning of the rice Na<sup>+</sup>/H<sup>+</sup> antiporter gene

**[0042]** A sequence having identity to Na<sup>+</sup>/H<sup>+</sup> antiporter (NHX1) obtained from budding yeast was analyzed from the database for higher plants of GeneBank. A cDNA clone from the cDNA library of rice panicle was identified. The amino acid sequence predicted from the clone had 37% identity with NHX1. It was presumed that the obtained cDNA clone did not have the full-length base sequence. Therefore, using the cDNA clone as a probe and using the cDNA library constructed from mRNA prepared from the root of rice (*Oryza sativa* L. cv Nipponbare) seedling as the template, selection of a cDNA clone having the full-length insertion was performed.

**[0043]** Rice seeds were imbibed overnight, and placed on cotton mesh suspended over a nutrient solution (0.5 mM NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 1 mM KNO<sub>3</sub>, 0.5 mM MgSO<sub>4</sub>, 12.5 μM Fe-EDTA, 1 mM CaCl<sub>2</sub>, micronutrients). Cultivation was performed 7 days with a cultivation condition: day(brightness 40 μmol m<sup>-2</sup> s<sup>-1</sup>) for 14 hours at 30°C, night for 10 hours at 25°C, humidity at 75%.

**[0044]** Poly (A<sup>+</sup>) RNA from the root of rice seedling was prepared and size fractionated by 5 to 25% sucrose density-gradient by centrifugation. Then, a cDNA library was constructed from the fractions containing relatively large poly (A<sup>+</sup>) RNAs (Tanaka, Y. *et al.* (1989) *Plant Physiol.* 90:1403-1407); Double stranded cDNA was synthesized from size fractionated poly(A<sup>+</sup>)RNA by the method of Gubler and Hoffman (Gubler, U. and Hoffman, B.J. (1983) *Gene* 25:263-269),

using oligo dT as the primer. The sample was then size fractionated by high performance liquid chromatography (Tosoh, Tokyo, model CCPD,) using Asahipack GS710 column (Asahi Chemical Industry Co. Ltd., Tokyo; 2.5 X 50 cm). cDNAs larger than 2kb were inserted to the EcoRI site of  $\lambda$ gt11.

[0045] Plaque hybridization was conducted using constructed  $\lambda$  phages having cDNA libraries, and cDNA clones that show identity with the NHX1 as probes. Selecting a vector with the longest cDNA insert from the plaques that showed signal, cloning was performed by inserting the cutout cDNA into a pBluescript (KS+) vector (Stratagene). Confirmation that the obtained cDNA clone is a full-length cDNA was made by the signal size from the Northern hybridization using RNAs extracted from the rice plant body and the obtained clone as the probe. All base sequence of the cDNA, to which the whole isolated gene (referred to as *OsNHX1*) is inserted, was determined (Figure 1).

[Example 2] Base sequence and amino acid sequence analysis of *OsNHX1* gene

[0046] The full-length sequence was 2330 base pairs, the 5' untranslated region was 296 base pairs, the translated region was 1608 base pairs and the 3' untranslated region was 426 base pairs. The protein encoded by *OsNHX1* was predicted to be 535 amino acids long, and the molecular weight was calculated to be 59,070 daltons. 59% of the predicted amino acids sequence was hydrophobic, 22% was neutral amino acids, and 19% was hydrophilic amino acids. Thus, the protein seemed to be highly hydrophobic. The result of hydrophobicity analysis, by the method of Kyte and Doolittle (Kyte, J. and Doolittle, R.F. (1982) *J.Mol.Biol.* 157:105-132), is shown in Figure 2. Twelve transmembrane regions were detected by the method of TMpred program (Hofmann, K. and Stoffel, W. (1993) *Biol.Chem. Hoppe-Seyler* 374:166).

[0047] Significant identity was detected for the amino acid sequence predicted from *OsNHX1* with the amino acid sequence of NHX1 and mammalian Na<sup>+</sup>/H<sup>+</sup> antiporter (NHE) (Table 1; NHX1 in the table represents that derived from yeast [*S.cerevisiae*], NHE6 from human, and NHE1 to 4 from rat. The values on the table were calculated by the homology-analyzing program (Lipman, D.J. and Pearson, W.R. (1985) *Science* 227:1435-1441) of GENETYX (ver.10) software (Software Development Company)). Especially high identity was observed in the transmembrane regions, which were suspected to be involved in ion transport (Figure 3). <sup>83</sup>LFFIYLLPPI<sup>92</sup>, a part of the amino acid sequence of *OsNHX1* (residues 83-92 of SEQ ID NO.2), is very well conserved in NHX1 and NHE and is expected to be the binding site of amiloride, an inhibitor of the eucaryotic Na<sup>+</sup>/H<sup>+</sup> antiporter (Counillon, L. *et al.* (1993) *Proc.Natl.Acad. Sci.USA* 90:4508-4512) (Figure 3A). In addition, the 6th and 7th transmembrane regions are well preserved in eucaryotic Na<sup>+</sup>/H<sup>+</sup> antiporter and, thus, is predicted to play an important role in the transport of Na<sup>+</sup> and H<sup>+</sup> (Orlowski, J. and Grinstein, S. (1997) *J.Biol.Chem.* 272:22373-22376). The 5th and 6th transmembrane regions in the amino acid sequence of *OsNHX1* showed high identity to these regions (Figure 3B). The above results indicate that the protein encoded by *OsNHX1* has the activity of Na<sup>+</sup>/H<sup>+</sup> antiporter.

Table 1

Amino acid sequence identity of <i>OsNHX1</i> to other Na <sup>+</sup> /H <sup>+</sup> antiporters (%)							
	<i>OsNHX1</i>	NHX1	NHE6	NHE1	NHE2	NHE3	NHE4
<i>OsNHX1</i>	100	29.5	33.0	30.1	29.4	26.7	27.7
NHX1		100	36.1	28.6	29.1	29.3	32.0
NHE6			100	31.9	29.1	31.8	28.6
NHE1				100	48.9	37.1	45.5
NHE2					100	44.7	66.0
NHE3						100	44.6
NHE4							100

[0048] Dendrogram of various Na<sup>+</sup>/H<sup>+</sup> antiporters reported to date, namely mammalian NHE, budding yeast (*S.cerevisiae*) NHX1 and NHA1, Sod2 (which is expected to be expressed on the plasma membrane of fission yeast, *S.pombe*), yeast (*Zygosaccharomyces rouxii*) ZSod3, *E.coli* NhaA and NhaB, as well as *OsNHX1* (noted as "*OsNHX1*" in the figures) made according to NJ method, revealed that three of them, that is NHX1, NHE6 and *OsNHX1*, form a cluster (Figure 4). It has been reported that NHX1 protein is expressed in the late endosome (Nass, R. and Rao, R. (1998) *J.Biol.Chem.* 273:21054-21060), and it was indicated that NHE6 protein is also expressed in the cell (Numata, M., Petrecca, K., Lake, N. and Orlowski, J., *J.Biol.Chem.* 273:6951-6959). Therefore, it is expected that *OsNHX1* protein is expressed in the intracellular organs, like the vacuole and so on, and plays an important role in Na<sup>+</sup> transport in these organs.



[Example 3] Production of transformed rice expressing rice Na<sup>+</sup>/H<sup>+</sup> antiporter gene

5 [0049] *OsNHX1* inserted in the *Bam*HI site of pBluescript KS+ (STRATAGENE) was excised with *Kpn*I and *Not*I. Then, *OsNHX1* was inserted downstream of the cauliflower mosaic virus 35S promoter of pMSH1 (for high expression) and pMSH2 (for repressed expression), both of which are derived from Ti-plasmid and are transferred with kanamycin resistance gene and hygromycin resistance gene (pMSH1: Kawasaki, T. *et al.* (1999) Proceedings of the National Academy of Sciences of the U.S.A. 96:10922-10926; pMSH2: the multi cloning site has the opposite direction compared to pMSH1). Using the constructed vector, the rice callus was transformed with *Agrobacterium tumefaciens*. The callus was induced from the seed, and screened after the infection with *Agrobacterium* was complete using hygromycin. 10 The screened callus was differentiated to obtain the transformant plant. Transformation and differentiation were basically performed according to the method of Toki (Toki, S. (1997) Plant Molecular Biology 15,16-21).

#### Industrial Applicability

15 [0050] According to the present invention, it is expected that isolated Na<sup>+</sup>/H<sup>+</sup> antiporter gene can render salt tolerance to the plant by expressing it in the plant. Therefore, it may conduce, for example, an increase in the harvest of crops, due to improvements in salt tolerance, by transfer into useful crops such as rice, which will make them resistant to harm by salt in dry land and such.

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SEQUENCE LISTING

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15

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20

25

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Cys Ala Cys Ile Val Leu Gly His Leu Leu Glu Glu Asn Arg Trp Val

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35

40

45

50

Asn Glu Ser Ile Thr Ala Leu Ile Ile Gly Leu Cys Thr Gly Val Val

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60

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485

490

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Thr Val His Tyr Tyr Trp Arg Lys Phe Asp Asp Ala Leu Met Arg Pro

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Met Phe Gly Gly Arg Gly Phe Val Pro Phe Ser Pro Gly Ser Pro Thr

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Glu Gln Ser His Gly Gly Arg

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**Claims**

1. A DNA selected from the group consisting of:

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- (a) a DNA encoding the protein consisting of the amino acid sequence described in SEQ ID NO: 2, and  
(b) a DNA comprising the coding region of the base sequence described in SEQ ID NO: 1.

2. A DNA encoding an Na<sup>+</sup>/H<sup>+</sup> antiporter derived from monocotyledoneae selected from the group consisting of:

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- (a) a DNA encoding the protein consisting of the amino acid sequence described in SEQ ID NO:2, wherein one or more amino acids are substituted, deleted, inserted and/or added, and  
(b) a DNA hybridizing under stringent conditions to the DNA consisting of the base sequence described in SEQ ID NO:1.

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3. The DNA of claim 2, wherein the monocotyledoneae is a plant belonging to the Gramineae family.

4. A vector comprising the DNA of claims 1 or 2.

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5. A transformant cell having the DNA of claims 1 or 2, or the vector of claim 4.

6. The transformant cell of claim 5, wherein the cell is a plant cell.

7. A protein encoded by the DNA of claims 1 or 2.

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8. A method for production of the protein of claim 7, which comprises the steps of:

cultivating the transformant cell of claim 5, and  
recovering the expressed protein from said cell or the supernatant of the culture thereof.

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9. A transformant plant comprising the transformant cell of claim 6.

10. The transformant plant of claim 9, wherein the plant is a monocotyledon.

11. The transformant plant of claim 10, wherein the plant is a plant belonging to the *Gramineae* family.

12. The transformant plant of claim 11, wherein the plant is rice.

5 13. A transformant plant that is the offspring or clone of the transformant plant of any of claims 9 to 12.

14. A material for the breeding of the transformant plant of any of claims 9 to 13.

10 15. An antibody that binds to the protein of claim 7.

16. A nucleic acid molecule that hybridizes with the DNA described in SEQ ID NO: 1, and which has a chain length of at least 15 nucleotides.

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Figure 1

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1  CAGAAGAGAGTTTCTAGCGAGCTCCGCGGAATCCGAGGCCAACCAGAGAGGCTCCGATACCAATCCCGATTCTCAACCTGATCCCCCCCCACGT
101 TCCTCGTTTCAATCTGTTCGCTCGGAATCGAATCTTTGTTTTTTTCTCTAATTTTACCGGAATTGTCGAATTAGGCATTACCAACGAGCAAGAG
201 GCGAGTGGATTGGTTGGTTAAAGCTCCGCATCTTGGCGCGGAATCTCGCTCTCTCTCTGCGGTGGGTGGCGGAGAGTCCCGCGCGTCCAGGCATGG
M G

301 GGATCGAGGTGGCGCGCGGCGGCTGGGGGCTCTGTACACGACCTCCGACTACGCGTGGGTGGGTCCATCAACCTGTTCTGCTCCGCGTCTGCGGCTG
H B V A A A R L G A L I T T S D Y A S V V S I N L P V A L L C A C

401 CATCGTCTCGCGCACCTCTCTCGAGGAGAAATCGCTGGCTCAATCACTCCATCAACCGGCTCATCATCGGGCTCTGCACCGCGGTGGTGATCTTGTGATG
I V L G H L L E E N R W V N E S I T A L I I G L C T G V V I L L M

501 ACCAAGCGGAAGAGCTCGCACTTATTCGCTCTCAGTGAGGATCTCTCTTCACTACCTCTCCCTCCGATCATCTTCAATGCAGGTTTTCAGGTAAGA
T K G K S S R L P V F S E D L P F I Y L L P P I I P N A G P Q V K K

601 AAAAGCAATCTTCCGGAATTTATGACGATCACAATTATTGGAGCGCTCGGGACAATGATATCTTTTACAAATATCTATTGCTGCCATTGCAATATT
K Q P P R N F M T I T L P G A V G T M I S P P T I S I A A I A I P

701 CAGCAGAAATGAACATTGGAAAGCTGAGTGTAGGAGATTTCTTGCAAATGGAGCCATCTTTCTGCGCAGAGATTCGTCTGCACATTCGAGGTCCTCAAT
S R M N I G T L D V G D P L A I G A I F S A T D S V C T L Q V L N

801 CAGGATGAGACACCCCTTTTGTACAGTCTGGTATTGGGTGAAGGTGTTGTGAACGATGCTACATCAATTGTGCTTTTCAACGCACTACAGAACTTGTATC
Q D E T P P L Y S L V P F G E G V V N D A T S I V L P N A L Q N F D L

901 TTGTCCACATAGATCGCGCTGTCTTCTGAAATCTTGGGGAACTCTTTTATTATTTTGTGCGAGCACCTTCTTGGAGTATTGCTGGATTGCTCAG
V H I D A A V V L K P L G N P F Y L F L S S T P L G V P A G L L S

1001 TGCATACATTAATCAAGAGCTATACATTGGAAGGCATTCTACTGACCGTGGAGGTCCCTTATGATGCTCATGGCTTCACTTATATATCTGCTGCTGAG
A Y I J K K L Y I G R H S T D R E V A L M H L M A Y L S Y M L A E

1101 TTGCTAGATTGAGCGGCATCTCACCGTATCTCTGCTGATTTGTAATGTCTCATTACACTTGGCATAACGTCACAGAGATTCAAGAGTTACACAA
L L D L S G I L T V P F P C G I V M S H Y T W H N V T E S S R V T T X

1201 AGCACGCTTTGCAACTCTGCTCTTCTGCTGAGACTTTTCTCTCTGATGTGGGATGGATGCATTGGATATGAAAAATGGAGTTTCCCACTGA
H A P A T L S P I A E T F L P L Y V G N D A L D I E K W E P A S D

1301 CAGACCTGGCAAAATCCATTGGGATAAGCTCAATTTTGTAGGATTGGTTCTGATTGGAAGAGCTGCTTTTCTATTCCCGCTGCTCTTCTTGTCCAACTA
R P G K S I G I S S I L L G L V L I G R A A P V P P L S P L S N L

1401 ACAAGAGAGCCACCGAATGAAAAATAACCTGGAGACAGCAAGTTGTAATATGGTGGCTGGGCTCATGAGAGGAGCTGTGCTGATTGCTCTTCTTACA
T K K A P N E K I T W R Q Q V V I W N A G L M R G A V S I A L A Y N

1501 ATAAGTTTCAAGATCTGGCCATACTCAGCTGCACGGCAATGCATAATGATCACCAGCACCATCAGTCTGCTTCTTTTACCACTATGCTATTGGGAT
K P T R S G H T Q L H G N A I M I T S T I T V V L P S T M V P G M

1601 GATGACAAAGCCATTGATCAGGCTGCTGCTACCGGCTCAGGCCATCTCTGACCTCTGAGCTTCAACCAAACTCCCTGCATTCTCTCTCTCTGACA
M T K P L I R L L L P A S G K P V T S E P S S P K S L H S P L L T

1701 AGCATGCAAGGTTCTGACCTCGAGAGTACAACCAACATTGTCAGGCTTCCAGCCCTCCGATGCTCTCACCAGCCGACCCACACTGTCCACTACTACT
S M Q G S D L E S T T N I V R P S S L R M L L T K P T H T V H Y Y W

1801 GCGCAAGTTCCAGACGCGGCTGATGCGACCGATGTTTGGCGCGCGCGGTTCGTGCGCTCTTCCCTGGATCACCACCGAGCAGAGCCATCGAGGAAG
R K P D D A L M R P M F G G R C P V P P S P G S P T E Q S H G R

1901 ATGACAGTGCACAAAGAAATGAAATGCAATGGTTGATGAGGAGAAATACATGTAATATGTACAGCAAAAGAGACAGCCCACTTTTGGCTTTGTACACTT
2001 TGGCTGCTGCTAATGATGCTTGTATAGTGCCTATATTCTTCAGAACTTCAGATGGTGCTCACCAGGCGCTTARGAGCCAGGAGGACCTTCTGATAATGGT
2101 TCGGGATGATGCTTTCTTCTGTCAGGATGAACCTTAGTGAGTGACACAGGGTGATGCTCTCCGACAACTGTAAATTTTGTAGATTACAGCCCACTTT
2201 GTACCTGTCTACCATCTTTAGTTGGCGGCTCTCTTCTAGTTGCCACCTGCAATGTAATGAAATCTCCGCCAAAATACATTTGTGTATATAA
2301 TTTTCTTCTGTTCAAAAAAAAAAAAAAAAAA

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Figure 2

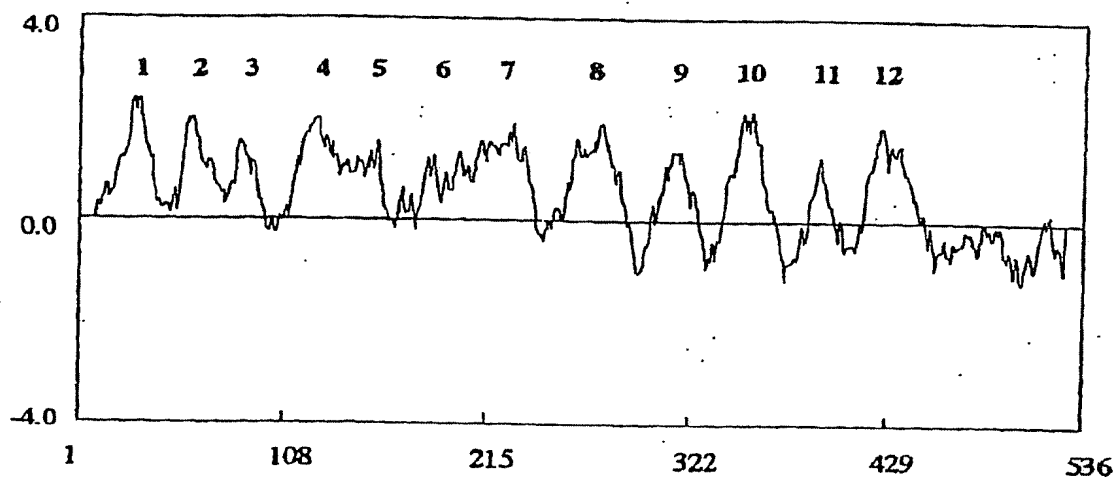




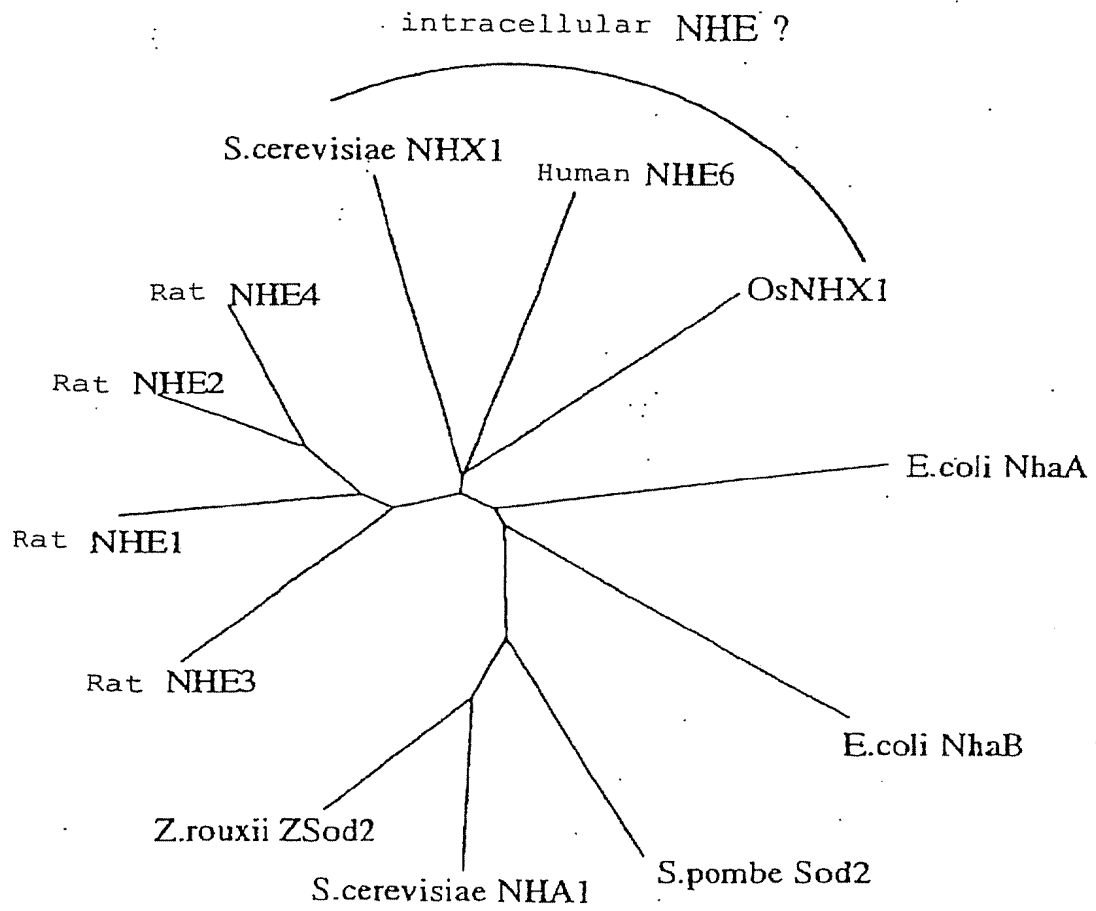
Figure 3

A	M3		M4	
OsNHX1	FSEDLFFIYLLPPIIFNAGFQVKKKQFFRNFM	TITLFGAVGTMISFFTISIAAIAIFSRM	138	
NHX1	FNSSYFFNVLLPPIILNSGYELNQVNFNNMLSILIFAIPGTFISAVVIGIILYIWTFLG	179		
NHE6	FDPEVFFNILLPPIIFYAGYSLKRRHFFRNLGSILAYAF	LGTATSCFVIGSIMYGCVTLM	205	
NHE1	LQSDVFFLFLPPIILDAGYFLPLRQFTENLGTILIFAVVGT	LWNAFFLGGLLYAVCLVG	219	
NHE2	MKTDVFFLYLLPPIVLDAGYFMPTRPFENLGTIFWYAVVGT	LWNSIGIGLSLFGICQIE	80	
NHE3	LTPTLFFFYLLPPIVLDAGYFMPNRLFFGNLGTILLYAVIGTIWNAATTGLSLYGVFLSG	166		
NHE4	MDSSLYFLYLLPPIVLESYGFMPTRPFENIGSILWWAGLGALINAFGIGLSLYFICQIK	184		
	:	: *	*****:	:*:: * *: * :. *: .

B	M5		M6	
OsNHX1	---NIGTLDVG---D	FLAIGAIFSATDSVCTLQVLNQDET-PFLYSLVFGEGVNDATSIV	192	
NHX1	----LESIDISFADAMSVGATLSATDPVTILSIFNAYKVDPKLYTIIFGESLLNDAISIV	235		
NHE6	KVTGQLAGDFYFTDCLLFGAIVSATDPVTVLAIFHELQVDVELYALLFGESVLNDAVAIV	265		
NHE1	---GEQINNIGLLDTLLFGSII	SAVDPVAVLAVFEEIHINELLHILVFGESLLNDAVTVV	276	
NHE2	---AFGLSDITLLCNLLFGSLISAVDPVAVLAVFENIHVNEQLYILVFGESLLNDAVTVV	137		
NHE3	---LMGELKIGLLDFLLFGSLIAAVDPVAVLAVFEEVHVNEVLFIIVFGESLLNDAVTVV	223		
NHE4	---AFGLGDINLLCNLLFGSLISAVDPVAVLAVFEEARVNEQLYMMIFGEALLNDGISVV	241		
	..	: : *	:*::*.* * :. .	*. :***:***. :*

Figure 4



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP99/07224

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> Int.Cl <sup>7</sup> C12N15/29, 5/14, C07K14/415, 16/16, C12P21/02, C12Q1/68, A01H5/00  According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) Int.Cl <sup>7</sup> C12N15/00-15/90  Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) GENBANK/EMBL/DBJ/GENESEQ SWISSPROT/PIR/GENESEQ BIOSIS (DIALOG), WPI (QUESTEL)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	Biochimica et Biophysica Acta, 1446 (1-2), p.149-155, 1999 July 7, Atsunori Fukuda et al., "Molecular cloning and expression of the Na <sup>+</sup> /H <sup>+</sup> exchanger gene in Oryza sativa"	1-16
P, X	Proc. Natl. Acad. Sci. USA, 96 (4), p.1480-1485, 1999 Feb. 16 Gaxiola, R.A. et al., "The Arabidopsis thaliana proton transporters, AtNhx1 and Avp1, can function in cation detoxification in yeast"	1-16
P, X	WO, 99/47679, A2 (BLUMWALD EDUARDO), 23 September, 1999 (23.09.99), Full text; Figs. 1 to 8 & AU, 9928214, A	1-16
A	J. Biol. Chem., 273, p.6951-6959, 1998 March 20 Numata M. et al., "Identification of a mitochondrial Na <sup>+</sup> /H <sup>+</sup> exchanger"	1-16
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 28 March, 2000 (28.03.00)		Date of mailing of the international search report 11 April, 2000 (11.04.00)
Name and mailing address of the ISA/ Japanese Patent Office		Authorized officer
Facsimile No.		Telephone No.

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP99/07224

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J.Biol.Chem., 267(13), p.9331-9339, 1992 May 5 Orlowski J. et al., "Molecular cloning of putative members of the Na/H exchanger gene family. cDNA cloning, deduced amino acid sequence, and mRNA tissue expression of the rat Na/H exchanger NHE-1 and two structurally related proteins."	1-16
A	J.Biol.Chem., 267, p.9340-9346, 1992 May 5 Tse C.M., et al., "Cloning and sequencing of a rabbit cDNA encoding an intestinal and kidney-specific Na(+)/H(+) exchanger isoform (NHE-3)"	1-16
A	Plant and Cell Physiology, 39(2), p.196-201, 1998 Feb. Fukuda Atsunori et al., "Na+/H+ antiporter in tonoplast vesicles from rice roots"	1-16
X	T. Sasaki, et al., "Rice cDNA from Panicle", Genbank accession, No.C91832, 20 April, 1998 (20.04.98),	16

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